Evaluation of Energy Dose and Output Power Optimum of Diode's Laser of 450 nm and 650 nm in Photoantimicrobial Mechanisms Against Inhibition of C. Albicans Biofilm Cells

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Submission date: 08-Nov-2018 03:25PM (UTC+0800)
Submission ID: 1035207159
File name: ion_of_Energy_Dose_and_Output_Power_Optimum_of_Diode_s_Laser.pdf (581.38K)
Word count: 4407
Character count: 22549

Journal of Physics: Conference Series

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Abstract. Photoantimicrobial as a pathogenic microbial inhibitory therapy system such as C. albicans in biofilms forms has been studied in vitro. Mechanisms of inhibiting called inactivating used the absorb principles of a dye agents such as chlorophyll against the photon energy of diode laser which any number of ROS product depend on energy doses of a laser, time of irradiation, concentration and time of incubation the dyes agent. The inactivation profile of C. albicans biofilm cells was observed based on cell viability reduction after photoantimicrobial treatment with or without oxygenation by *XTT assay* test. Results show that the inhibiting significantly with the time incubation of the dye agents and the oxygen degree inside the sample. The inhibition for oxygenation biofilm's group 10% lower than without oxygenation biofilm's group at the maximum of reduction of cell viability occurred in the 3-hour incubation group. Optimum of inactivation are 89.6% (without oxygenation) and 94.8% (with oxygenation) after irradiation with 450 nm laser (power output 128.73 at energy dose 86.09 J/cm^2), While, by 650 nm laser (power output 164.53 mW at energy dose 92.52 J/cm^2) irradiation treatment obtained optimum of inactivation are 89.5% (without oxygenation) and 92.3% (with oxygenation).

1. Introduction

The photodynamic therapy known as PDT began to be studied in 1900 by Oscar Raab which saw the effects of light on dyestuffs in medicine, followed by Lipson and Schwartz in 1960 which found a neoplasm lesions emit fluorescence light during surgery after *hematoporphyrin* injection [1]. Initially, PDT therapy was studied to kill cancer cells (malignant cells) but has evolved and proven effective against infectious diseases caused by pathogenic microbes known as *antimicrobial photodynamic therapy* (aPDT) which popular kind photoinactivation [2].

In the photonic, aPDT mechanisms are therapy that involves interaction between light and biomolecule and throught photophysics process and photochemistry process like absorbtion of photon energy, electronic transition, energy or electron transfer against other molecule, sustainable of chemical reaction and produce the radical compounds. The radical compounds produced in the aPDT mechanism can be superoxide anions, hydrogen peroxide, and hydroxyl radicals or oxygen singlet

(called *reactive oxygen species* (ROS)) that property very reactive and toxic, that believed can be killing the pathogen microbial cell [3,4,5].

The principle of the aPDT mechanism is different from that of specific antibiotic therapy because inhibiting process on the aPDT therapy is inactivating cells division system and break DNA chain the cells that active by ROS. There are three pathways of photoinactivation to microbes are 1) light is absorbed by a sensitizer outside the cell and forms a ROS that will be lysing the cell membrane; 2) the light who penetrates the cell wall and is absorbed by the chromophore inside cell and forms another ROS; 3) the light which directs inside cell breaks the DNA chain [6].

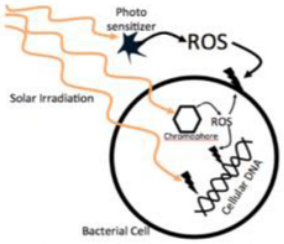


Figure 1. Three pathways for bacterial photoinactivation [6].

A component in a target molecule that acts as a photon light absorber called a chromophore, generally in the form of a molecule called photosensitizer (PS). PS can be from target cell self (endogenous PS) or can be from outer of target cell (exogenous PS) that added on target cell before irradiating. The success of the aPDT mechanisms depends on the suitability between the wavelength of light and the optimum uptake of chromophores to light and the presence of oxygen molecules around the cell target. Types of PS that have been shown to be effective as sensitizers in photoinactivation [7-11] include methylene blue, toluidine blue, phthalocyanine, protoporphyrin IX and deuteroporphyrin IX. In this study used chlorophyll as PS because it has the characteristics absorbtion optimum at two wavelength area (413 nm and 650 nm) [12] so that it can be combined with different source of light. Chlorophyll molecule is PS of the third generation applied on PDT because of their properties as a pure chemical compound, maximum absorbance below 650 nm, a high-value yield to produce ROS as well as long lifetime, no toxic in dark, stable and easily isolate [13,14]. Excess chlorophyll as a sensitizer is also used in photoinactivation of pathogenic microbes because many plants in Indonesia have active substances as antimicrobials.

The increasing problem of pathogenic microbial cell resistance to antibiotic drug types and the antibiotic drugs treatment's inconsistently by patient causing aPDT mechanisms as a promising alternative therapy. aPDT studies have been widely developed to inactivate pathogenic microbes especially oral infections caused by C. albicans microbes. The pathogenicity of C. albicans is caused by microbial characteristics such as dimorphism and biofilm formation. Characteristics of biofilm C. albicans is the main object will be investigated through the application of aPDT mechanism.

The light source will be used on aPDT must is known the output power characteristics and exposure area. This characteristic is important for determining the irradiation dosimetry which will be adjusted with the absorption characteristics of the photosensitizers will be used. In this research, used two types of the laser diode with a specification of wavelength 450 nm and 650 nm, for determining optimum spectrum that effective to inhibit the growth of C. albicans biofilm cells. The irradiating parameter of the light source to be determined as the characteristic of the light source is the stability of the power against change of time and distance, the intensity and exposure area, the energy dose of irradiation. The analysis conducted in this study is the wavelength spectrum and the energy dose of irradiation that optimum is effectively reduced the viability of C. albicans cells through optical density measurement by XTT assay method.

2. Experiment

Papaya leaf chlorophyll extract was isolated by maceration with a mixture of methanol solvent, petroleum ether and diethyl ether which was further fractionated with n-hexane and acetone [12]. The maximum absorbance characteristics of chlorophyll extracts obtained at two wavelengths were 405 nm (Soret band) and 650 nm (Q-band) and the MIC values obtained were 0.5 $mg.mL^{-1}$, 3.75 $mg.mL^{-1}$ and 5.0 $mg.mL^{-1}$.

The laser diode fabrication specifications used with a power output of 100 mW, subsequently characterized in advance to determine the proper research design. The diode laser characterization test includes optimum output power to wavelength (monochromator JASCO CT-10), exposure time, change of radiation distance (PM100D Thorlabs Optical Power Meters), light intensity, exposure area and radiation dose, whereas PS used is a chlorophyll molecule isolated from papaya leaf and had been tested for characteristics with fluorescence spectroscopy and UV- Vis Shimadzu.

The optimum characteristic results are adjusted for the duration of irradiation set to determine the radiation dose as the treatment variation. The prescribed radiation time is 60 s, 120 s, 180 s, 240 s, and 300 s, while the duration of PS incubation before aPDT is applied is 1 hour and 3 hours.

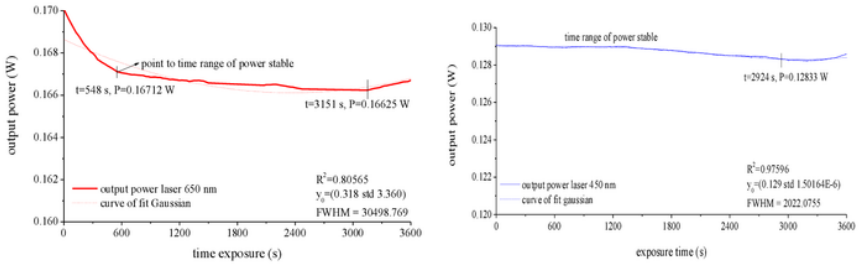
Biofilm C. albicans used as research object was grown for 6 days in a 96-well microplate container. Inocula C. albicans 0.5 McF in sterile PBS pH 7.4 was planted in each well as 100 µL then incubated for 90 min in a 37^0C incubator shaker, 120 rpm. The well was washed with sterile PBS pH 7.4 twice and added BHI-B 8% glucose into each well as 200 µL. The biofilm suspension was incubated in a 37^0C incubator shaker, 120 rpm for 6 days. At harvest, microplate contents are poured over a container containing tissue to remove C. albicans planktonic cells that do not form biofilms. 200 µL chlorophyll extracts of various concentrations were injected on the biofilm of C. albicans in a predetermined well (the biofilm group using the sensitizer as the treatment).

As a comparison of the aPDT treatment, in the biofilm of C. albicans before the chlorophyll extract was first extracted oxygen gas for 30 seconds with flowrate variation of 1 $L.min^{-1}$ (80 psi), 2 $L.min^{-1}$ (70 psi), 3 $L.min^{-1}$ (60 psi) and 4 $L.min^{-1}$ (50 psi) at pressure adjusted to the regulator scale listed for each oxygen gas stream (pressure decreases as oxygen exits from the tube). The oxygenation mechanisms performed to overcome the limitations of the oxygen zone in the biofilm. Irradiated biofilms (microplates with laser treatment groups only and laser-sensitizer combinations) are placed under pre-prepared laser devices, and light exposure during the time set for each appropriate well. Each well-treated microplate-96 wells treated for all treatment groups were washed with sterile PBS pH 7.4 twice subsequently stained 40 µL XTT (1 $mg.mL^{-1}$) + 2 µL menadione (10 $mg.mL^{-1}$) + 158 µL sterile PBS pH 7.4 and incubated for 2 hours. 100 µL result of staining transfer to a new microplate for readability of viability cells with ELISA READER.

3. Result and discussion

The diode laser characteristic from both of laser (450 nm and 650 nm) that result in the maximum of power respectively is 35,790 mW at 445 nm wavelength (red laser) and 45,499 mW at 650 nm wavelength (blue laser). This output power is the true power value of both lasers based on the determination of each wavelength using a monochromator.

The power stability test against to the exposure time is measured to see power stability for one hour operating. It is important to determine the length of exposure that still produces stable power.



(a) (b)

Figure 2. Characteristic of power stable against time exposure of laser 650 nm (red) and 450 nm (blue).

Figure 2 shows that blue laser relative more stable than the red laser. The blue laser stable starting from the time start and increasing after 2924 s exposure time with a stable power of 0.12833 W, while the red laser has a stable area after exposure time 548 s with a stable power of 0.16712 W.

These research did not use a light filter adjusted to the maximum absorbance of the sensitizer so that the possibility of photosensitization for all range wavelengths absorbance of papaya chlorophyll allowed it to occur. The light photon energy coming out of each laser is $3x10^{-19}$ J (laser 650 nm) and $4.47x10^{-19}$ J (450 nm laser).

The characteristic of a stable power to change in distance is determined by adjusting the distance between samples with light from a distance of 1 cm to 5 cm. The measurements of the two lasers obtained different distances of light samples at maximum power, where the measurements for the red laser, optimum power occurred at a distance of 3 cm (power $0.16453\pm4.22\times10^{-6}$ W) whereas for blue laser, optimum power occurred at a distance of 1 cm (power $0.12873\pm1.17\times10^{-5}$ W). Theoretically, the further the distance between the light source and the sample, the strength will remain stable but the intensity changes as the exposure area also changes. When compared to a distance of 5 cm (power $0.15839\pm5.2\times10^{-6}$ W for the red laser) and a distance of 2 cm (power $0.12790\pm2.1\times10^{-5}$ W for the blue laser) can be said laser power remains stable.

The result of extensive measurement laser beam, the intensity of the laser light, exposure time and dose of radiation energy as a variable dosimetry in aPDT for any distance corresponding to the power stability test against more distance shown in table 1. The result of measuring the area of irradiation for a red laser at a distance of 3 cm is 0.453 cm^2 while for blue laser 0.357 cm^2. Intensity at 3 cm distance of is 0.363 $W.cm^{-2}$ (red laser) and 0.360 $W.cm^{-2}$ (blue laser). Value of intensity to be reference for determining the energy dose yang sampai di permukaan biofilm.

Table 1. Dosimetry of aPDT C. albicans biofilm mediated *carica papaya* L. chlorophyll extract with energy dose difference for both laser .

No	time irradiance (s)	energy of dose ($J.cm^{-2}$)	
		650nm	450nm
1	60 (L1)	28.70	30.84
2	120 (L2)	57.39	61.68
3	180 (L3)	86.09	92.52
4	240 (L4)	114.78	123.36
5	300 (L5)	143.48	154.20

Results aPDT treatment for all treatment groups: control group, sensitizer group only, only the laser groups and the combination group laser-sensitizer in three variations concentration of sensitizer, time of sensitizer incubation and energy dose irradiation.

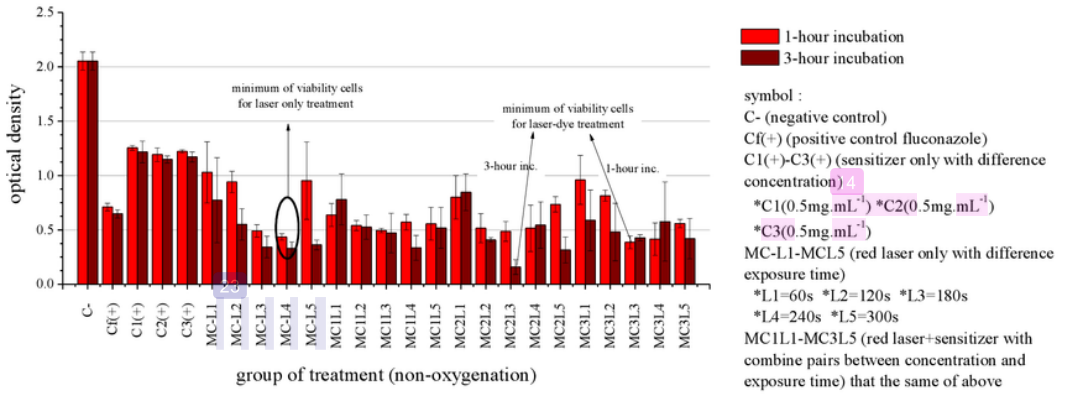


Figure 3. Viability C. albicans biofilms cell for 650 nm laser treatment.

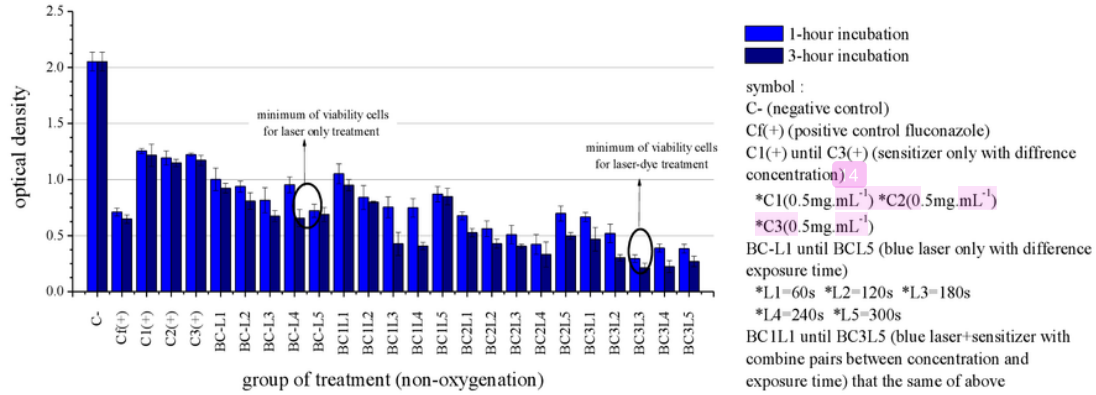


Figure 4. Viability C. albicans biofilms cell for 450 nm laser treatment.

Figures 3 and 4 show the results of optical density reading of aPDT treatment results on C. albicans biofilm cells without oxygenation respectively for 650 nm lasers (figure 3) and for 450 nm lasers (figure 4) at significance ($p>0.05$). In general, the laser only groups are more effective than the sensitizer only groups and the chlorophyll mediation laser groups is most effective than another group (groups of sensitizers only or laser only). The effect of inactivation of the red laser can reduce viability until 92% than the blue laser. The effect of time of incubation also showed significant results where 3-hours of incubation more effective than time 1-hour of incubation. The concentration effect of chlorophyll does not show a uniform pattern, which theoretically, higher concentration correlating with the greater absorption of light photons thus leads to more opportunities for the formation of radical compounds that will affect to be success of inactivation. Figure 3 shows the maximum reduction occurring in the MC2L3 group (1-hour incubation) and in the MC3L3 group (3-h

incubation). Figure 4 shows the maximum reduction occurring in the same group is the BC3L3 group for different the time of incubation.

The optimum inactivation with the red laser irradiating occurs at doses of 86.09 J.cm^{-2} for both time incubation of sensitizer with percent inactivation obtained respectively 81.2% (1-hour incubation) and 92.3% (3-hours incubation). Both inactivation optimum value is obtained from the treatment with the sensitizer concentration of each 3.75 mg.mL^{-1} and 5.0 mg.mL^{-1}. The result of optimum inactivation with the blue laser irradiating obtained 85.6% (1-hour incubation) and 89.6% (3-hour incubation) at an energy dose of 92.52 J.cm^{-2}.

For the additional treatment with oxygenation mechanisms, see figure 5 below. The condition of treatment which applied on with oxygenation groups only the group with optimum inactivation i.e concentration of PS is C3=5.0 mg.mL^{-1} at 3-hour incubation.

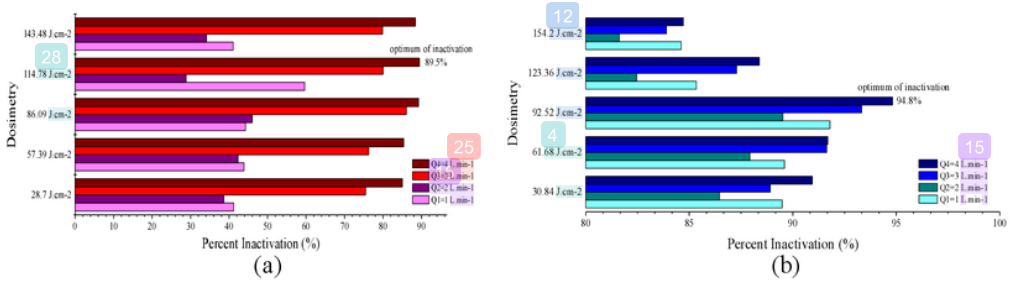


Figure 5. Inactivation of 650 nm laser based on dosimetry (energy dose) with oxygenation mechanisms on C. albicans biofilm (a) red laser treatment; (b) blue laser treatment.

The highest of percent inactivation for red laser (650 nm) induction is for flow rate Q4 with the energy of dose 114.78 J.cm^{-2} is 89.5% and the lowest value for flow rate Q2 with the same energy of dose is 28.8%. The highest of percent inactivation for red laser (450 nm) induction is for flow rate Q4 with the energy of dose 92.52 J.cm^{-2} is 94.8% and the lowest value for flow rate Q2 with the same energy of dose 154.20 J.cm^{-2} is 81.6%.

Before used, laser (light source) must be performance tested to determine the dosimetry to be applied in aPDT. In dosimetry of light, total energy be expressed in units of Joule that depend of exposure time and power of light. Intensity are fluence rate is expressed in W/area. The fluence rate should not more than 0.200 W/cm2 (for microlens output light) and 0.400 W/cm2 (for cylindrical diffuser) because more than the value specified can be damaging cell normal that caused by the thermal effect. The laser that used in this study still include safe.

The time incubation of sensitizer has a different effect against C albicans biofilms cell because of antifungal properties of a sensitizer. The longer the incubation of the sensitizer before the treatment, the more chlorophyll substances that diffuse into the biofilm layer. In general, increasing the dose of irradiation energy inconsistent with increasing percentage of inactivation. It is assumed that when treatment and staining can be affected by human errors such as inhomogeneous exposure at the sample and the long exposure can decrease laser output power so that the energy of dose be different with the calculation result. Another difference between theory and practice is the time of staining which using three components of the reagent wherein one component of menadione (in a very small volume scale of 2 µL) is not expected to be sucked by a micropipette or while injection can exceed the volume so that sample has more or few dense coloration.

Figures 5(a) and 5(b) represent inactivation profiles of C. albicans biofilm cells with additional treatment being oxygen streaming in order to increase oxygen levels in biofilm inner layers. With the pressure and flow of certain flow of oxygen can diffuse to a certain depth and even have the opportunity to reach the bottom of the biofilm in the well. The inactivation results showing percent

inactivation significant with the increase of flow and pressure, where for the Q1 and Q2 although at low volume but flowed at high pressure, while for the Q3 and Q4 even at low pressure but the volume of oxygen relatively higher.

4. Conclusion

The percent value of inactivation from the aPDT treatment results from the second treatment of different wavelengths and power shows significantly different results. In the no oxygenated sample group, the inactivation percent for laser treatment was 650 nm higher than for the 450 nm laser treatment. In the sample group with an oxygenation mechanism prior to the aPDT treatment, the inactivation effect of the laser 450 nm was more effective than the inactivation effect of the 650 nm laser. For 450 nm laser irradiation with photon energy is 4.47×10^{-19} J and power output 0.12873 W, optimum inactivation is 89.6% (without oxygenation) and 94.8% (with oxygenation) at energy dose 86.09 J.cm^{-2}. At an irradiation of 650 nm laser with photon energy 3×10^{-19} J and power output 0.16453 W, optimum inactivation is 89.5% (without oxygenation) and 92.3% (with oxygenation) at energy dose 92.52 J.cm^{-2}.

Acknowledgments

Thanks to all those who have assisted in the completion of this research, so that all the data in this paper is eligible to be published. Especially the party who has sponsored this research through doctoral dissertation grant.

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